Docket No.: TJU0006-101 **PATENT APPLICATION**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Waldman, S.A. *et al.*

Serial No.: 10/695,578 **Group Art Unit: 1642**

Filed: October 27, 2003 **Examiner: Aeder, Sean E.**

Title: Metastatic Colorectal Cancer Vaccine

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

DECLARATION OF DR. SCOTT A. WALDMAN UNDER 37 CFR 1.132

I, Scott A. Waldman, M.D., Ph.D., do hereby declare:

1. I am the inventor of the subject matter claimed in the above-identified patent application.

2. Experiments were performed by me or by others in my laboratory under my supervision to test an anti-colorectal cancer vaccine using GCC sequences as antigenic targets.

3. Recombinant adenovirus expressing secreted forms of the GCC extracellular domain (GCCECD) were generated (GCCECD-AV). Also, forms of the GCCECD tagged with either glutathione-S-transferase (GST) or hexahistidine (hexahis) were introduced into bacterial or mammalian expression systems, respectively, and purified. Additionally, the truncated transmembrane form of GCC (GCCTM) was stably introduced into MC38 (C57BL/6) and CT26 (BALB/c) mouse colon cancer cells. Full-

length GCC was not used because the intracellular signaling domain inhibits proliferation of human and mouse cells. GCCTM expressed by mouse cells retained robust ligand binding, which is dependent on normal glycosylation.

4. Recombinant adenovirus expressing secreted forms of the GCC extracellular domain (GCCECD) were generated using the Adeno-X Virus Purification Kit (Clontech) and titered using the Adeno-X Rapid Titer Kit (Clontech) according to manufacturer's instructions. LacZ-AV was acquired from Clontech (LacZ-Adeno-X), expanded, purified and titered in parallel with GCCECD-AV. Also, GCCECD tagged with glutathione-S-transferase (GST) was expressed and purified using B-Per Bacterial Protein Extraction Reagent (Pierce) from a bacterial expression system in the plasmid pGEX-4T-1. Hexahistidine- (hexahis) tagged GCCECD was purified with Ni-NTA agarose (Qiagen) from supernatants of GCCECD-AV-infected 293A cells. Additionally, the truncated transmembrane form of GCC (GCCTM) was stably introduced into MC38 (C57BL/6) and CT26 (BALB/c) mouse colon cancer cells as GCC-expressing tumor models, using the retroviral plasmid pMSCV2.2-Puro and selected with puromycin. GCCTM containing a pentahistidine tag inserted between the signal peptide and ligand-binding domain was generated by PCR. The pentahistidine tag does not interfere with membrane-trafficking or ligand binding of GCCTM but allows detection by extracellular staining with anti-pentahistidine antibody and flow cytometry.

5. Mice were immunized with GST-GCCECD using complete Freund's adjuvant during priming and incomplete Freund's adjuvant during boosting. Also, mice were immunized with a purified GST-fusion protein as a negative control. Seven days after boosting, CD4+ T cells were purified from total splenocytes using magnetic cell sorting (Miltenyi Biotec) and stimulated ex vivo with purified hexahis-GCCECD or GST protein (Sigma) and splenocytes as a source of antigen-presenting cells. CD4+ T cell responses

were detected by 3H-thymidine. The specificity of proliferative responses was confirmed using CD4+ T-depleted fractions (CD4-) and T cells from animals immunized with GST-control protein.

6. Mice were immunized with GCCECD-AV or LacZ-AV as a control. Ten days after immunization, splenocytes were collected and CD8+ T cells were purified from splenocytes using magnetic cell sorting (Miltenyi Biotec). CD8+ T cells were stimulated ex vivo with LacZ- or GCCECD-expressing MC38 cells. Responses were detected by ELISpot assay for IFNγ-producing cells, employing anti-IFNγ antibodies (Pharmingen) and alkaline phosphatase-conjugated streptavidin and NBT/BCIP substrate (Pierce). Specificity was demonstrated by the absence of IFNγ production following stimulation of GCC-immunized T cells by stimulator cells lacking GCC or following stimulation of control-immunized T cells by stimulators expressing GCC.

7. C57BL/6 mice were immunized with GCCECD-AV or a control adenovirus. Seven days later mice were challenged by subcutaneous injection with MC38 mouse colorectal cancer cells stably expressing recombinant GCC extracellular domain on the surface (GCCTM). Tumor volumes (mm^3) were calculated using the following equation after measuring three orthogonal diameters with calipers: $4/3\ \pi$ r1 r2 r3 where (r) is half the diameter.

8. BALB/c mice were immunized with GCCECD-AV or a control adenovirus. Seven days later mice were injected by tail vein with the murine colon cancer cell line CT26 stably expressing recombinant GCCTM. Mice were sacrificed 14 days later, lung parenchyma stained with India ink, and the white metastatic nodules quantified in each animal.

9. GCC induced robust antigen-specific T cell responses. GST-GCCECD-immunized C57BL/6 mice produced an antigen-specific CD4+ T cell response compared to mice immunized with a control GST fusion protein, measured by proliferation upon ex vivo stimulation with purified hexahis-GCCECD. Proliferative responses were CD4+ T cell-specific since purified CD4+ T cells, but not cell preparations depleted of CD4+ T cells, incorporated 3H-thymidine. Moreover, responses were antigen-specific since neither purified CD4+ T cells, nor cell preparations depleted of CD4+ T cells obtained from GST-control immunized mice incorporated 3H-thymidine in response to GCCECD. Furthermore, CD8+ T cells purified from mice immunized with GCCECD-AV, but not LacZ-AV, exhibited an IFNγ response upon stimulation by cells expressing GCCECD, but not those expressing LacZ. Conversely, CD8+ T cells obtained from mice immunized with LacZ-AV, but not GCCECD-AV, exhibited an IFNγ response upon stimulation by cells expressing LacZ, but not GCCECD.

10. Results showed that GCC is a tumor rejection antigen in subcutaneous and parenchymal mouse isograft models. C57BL/6 mice were immunized once with GCCECD-AV or control AV, followed by subcutaneous inoculation of MC38 mouse colon cancer cells expressing GCCTM. Growth of GCCTM-expressing MC38 tumors was delayed ~50% in GCCECD-AV compared to control immunized mice. While the subcutaneous graft model is advantageous because it permits longitudinal evaluation of tumor growth kinetics by direct volumetric quantification, the most common sites for metastatic colon cancer in patients are parenchymal tissues, including lung. Thus, BALB/c mice were immunized once with GCCECD-AV and one week later mouse CT26 colon cancer cells expressing GCCTM were administered by tail vein to establish lung metastases. In this model, GCCECD-AV, but not control AV, immunization nearly prevented the development of tumor metastases in lung. These observations support the conclusion that GCC produces immunological responses effective against metastatic

tumors. It is noteworthy that only a single GCCECD-AV immunization was required to achieve tumor immunity and prevention of parenchymal metastases. Moreover, the generalizability of this immunological approach is suggested by the anti-tumor efficacy of GCC immunization in two strains of mice (C57BL/6, BALB/c), employing different colon cancer cell lines (MC38, CT26), and different isograft models (subcutaneous, parenchymal).

11. I hereby declare that all statements made herein are true and that all statements made n information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the applications and any patent issued thereon.

Date:

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Scott A. Waldman, M.D. PhD